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(54) Title: FELINE IMMUNODEFICIENCY VIRUS VACCINE (57) Abstract <p>Disclosed are vaccines containing both a DNA sequences encoding FIV <i>gag</i> protein and a DNA sequence encoding FIV <i>env</i> protein. The <i>gag</i> and <i>env</i> proteins are preferably expressed by baculovirus expression systems containing the DNA sequences encoding the FIV <i>env</i> and <i>gag</i> proteins or in feline herpes virus vectors containing the DNA sequences encoding the FIV <i>env</i> and <i>gag</i> proteins. Also disclosed are combined mucosal/parenteral, mucosal/mucosal and parenteral/parenteral inoculation methods.</p>		

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FELINE IMMUNODEFICIENCY VIRUS VACCINE

Field of the Invention

The present invention relates generally to the fields of immunology and
5 recombinant genetics. More specifically, the invention relates to a recombinant
vaccine which includes both the DNA sequences encoding the viral envelope and *gag*
proteins of feline immunodeficiency virus and methods of using vaccines based on
these encoded DNA sequences.

Background of the Invention

10 Feline immunodeficiency virus (FIV), formerly called feline T lymphotropic
lentivirus, was first isolated in 1986 from a large multiple cat household in
Petaluma, California (Pederson et al., Science (1987) 235:790). FIV has been
classified as a member of the subfamily *Lentiviridae* in the family *Retroviridae*.
This is the family that includes human and simian immunodeficiency viruses,
15 equine infectious anaemia, maedi visna of sheep and caprine-arthritis encephalitis
viruses (CAEV). The genome of FIV is organized like other lentiviruses with three
long open reading frames corresponding to *gag*, *pol* and *env* (Talbot et al., Proc.
Natl. Acad. Sci. (1989) 86:5743; Olmsted et al., Proc. Natl. Acad. Sci. (1989)
86:2448). The *gag* gene codes for the major structural components of the virus, the
20 *env* gene codes for the envelope glycoprotein, and the *pol* gene codes for the
polymerase protein.

The *gag* gene is expressed as a 50 kD polyprotein which is processed into
three subunits: a p15 matrix protein, a p24 capsid protein, and a p10 nucleocapsid
protein. The *pol* gene encodes three proteins: the protease, reverse transcriptase
25 and a p14.6 protein of unknown function. Autoprocessing by the protease portion of
the gene gives rise to all three proteins of the *pol* region. Additionally, the protease
is responsible for the processing of the *gag* precursor. The *pol* gene is expressed as
a *gag-pol* fusion protein. The envelope gene is expressed as a 160 kD glycoprotein,
gp160. The antigenicity of the FIV core proteins is similar to other lentiviruses.

30 Surveys indicate that the median age of FIV infected cats in the general
population is about 3 years, whereas the median age of clinically diseased FIV
infected cats is about 10 years of age (Shelton et al., J. Am. Anim. Hosp. Assoc.
(1989) 25:7). The clinical sequela of FIV infection in cats has been divided into five
stages. Stage I, the primary phase, is quite variable with some animals showing
35 degrees of fever, neutropenia, generalized lymphadenopathy, diarrhea and
depression. Animals at this stage are normally not recognized by owners to be ill

and the signs listed above have been variably recognized in experimental infections. Mortality is low and, despite recovery from this phase, virtually all cats become lifelong carriers. Viremia is by far and away the most consistent sign associated with this phase.

5 During Stage II, virus can still be found in the blood of cats and it is now that major abnormalities evolve in circulating numbers of CD4+ and CD8+ T cells. It is during Stage III that many cats are first presented to veterinarians with signs which range from vague clinical symptoms like recurrent fever, weight loss and anorexia, to animals which show obvious indications of chronic secondary or
10 opportunistic infections which can include chronic oral cavity infections, chronic enteritis, chronic respiratory infections, chronic conjunctivitis and bacterial infections of the urinary tract and skin. Stage IV is dominated by the chronic secondary infections described in Stage III with further weight loss and hematological abnormalities. Finally, in Stage V, the health of cats declines further
15 over a period of months to years and a few surviving animals may develop a condition analogous to human acquired immune deficiency syndrome (AIDS) with opportunistic infections at multiple body sites.

The virus replicates optimally in blood mononuclear cells and has a tropism for T lymphocytes, peritoneal macrophage, brain macrophage and astrocytes. In
20 common with other retroviruses, the genetic material of FIV is composed of RNA and the production of a DNA copy of the viral RNA is an essential step in the replication of FIV in the host. This step requires the enzyme reverse transcriptase which is carried into the host by the invading virus. The DNA version of the viral genome is inserted into the genetic material of infected host cells in which it
25 continues to reside as a provirus. This provirus is replicated every time the cell divides and can code for the production of new virus particles. Cells infected with FIV remain infected for the duration of their lifespan.

The virus appears to be spread by horizontal transmission, predominantly by bite wounds from an infected cat as these animals shed appreciable amounts of
30 virus in saliva (Yamamoto et al., Am. J. Vet. Res. (1988) 8:1246). Vertical transmission has been reported, but is rare. Given this mode of transmission, it is theoretically possible to give a measure of protection with antibody.

At the present time there are no vaccines commercially available which provide protection against infection with FIV. Recent work suggests that cats
35 immunized with whole infected cells or cell-free virus are protected against challenge and that protection correlated in large degree to the presence of serum

neutralizing antibodies (Yamamoto et al., J. Virol. (1993) 67:601; Yamamoto et al., AIDS Res. Hum. Retroviruses (1991) 7:911. A disadvantage of these systems, however, is that the use of inactivated vaccines does not completely rule out iatrogenic transmission of FIV. Another disadvantage of using inactivated
5 preparations is that they generally do not provide correct FIV antigen presentation for cytotoxic T cell development. Other investigators have focused on recombinant approaches to develop an FIV vaccine, either in the form of subunit or viral vectored vaccines. PCT patent application WO 92/15684 represents such an effort.

PCT patent application WO 92/15684 reports the cloning and expression of
10 glycoprotein (gp) 160 envelope protein, gp120 envelope protein and p24 *gag* protein from FIV and suggests that these proteins are useful in the diagnosis, treatment and prevention of FIV. Specifically, it suggests using recombinant proteins from the gp160 envelope protein or the *gag* protein to develop vaccines in the prevention of FIV infection in cats. Abstracts of presentations made by the inventors of WO
15 92/15684, or their collaborators, at the International Symposium on Feline Retrovirus Research, Research Triangle Park, North Carolina, USA, October 6-9, 1993, reveal that while their recombinant FIV *env*-gene product induces high ELISA antibody titers, these high titers do not correlate with virus-neutralization or with protection from FIV infection.

20 Thus, there remains a continuing need for a recombinant vaccine which yields a non-infectious FIV product and takes away the necessity of inactivating FIV-infected whole cell vaccines while providing good levels of protection. In addition to this prophylactic use, vaccines may also be useful for post-exposure immunotherapy. For example, current rabies vaccines are given to individuals
25 following potential exposure to rabies viruses. Since FIV has a long period of latency between infection and disease progression, immunotherapy could also be of value in arresting the progression of FIV disease in infected cats.

We describe here the construction of recombinant baculoviruses and recombinant feline herpes viruses that express the *gag* and *env* genes of FIV. In
30 addition, the present invention provides means for making FIV vaccines based on these recombinant viruses. The invention also includes methods of vaccinating a mammal by administration of a vaccine to both mucosal and parenteral sites in the natural viral host to achieve essentially full protection against persistent viraemia.

Information Disclosure

35 Cole GE et al., J. Virol. (1990) 64:4930, report the expression of feline leukemia virus envelope and *gag* proteins in recombinant feline herpesviruses.

Olmsted et al., Proc. Natl. Acad. Sci. (1989) 86:2448, report the molecular cloning of feline immunodeficiency virus.

Olmsted et al., Proc. Natl. Acad. Sci. USA (1989) 86:8088, report a nucleotide sequence analysis of feline immunodeficiency virus and discuss the genome

5 organization and relationship of FIV to other lentiviruses.

Pederson et al., Science (1987) 235:790, report the isolation of a T lymphotropic virus from domestic cats with immunodeficiency-like syndrome.

Shelton et al., J. Am. Anim. Hosp. Assoc. (1989) 25:7, report the prevalence of feline immunodeficiency virus and feline leukemia virus infections in pet cats.

10 Talbott et al., Proc. Natl. Acad. Sci. (1989) 86:5743, report the nucleotide sequence and genomic organization of feline immunodeficiency virus.

Yamamoto et al., J. Virol. (1993) 67:601, report the experimental protection against homologous and heterologous strains of feline immunodeficiency virus.

15 Yamamoto et al., AIDS Res. Hum. Retroviruses (1991) 7:911, report the experimental vaccine protection against feline immunodeficiency virus.

Nunberg et al., J. Virol. (1989) 63:3240, report the creation of a feline herpes virus (FHV) thymidine kinase deleted vector.

Hu et al., Science (1992) 255:456, report the protection of macaques against SIV infection by subunit vaccines of SIV envelope glycoprotein gp160.

20 Lutz et al., "Vaccination of Cats with Recombinant FIV *env*-gene Products," International Symposium on Feline Retrovirus Research, Research Triangle Park, North Carolina, USA, October 6-9, 1993, report that while it is relatively easy to induce high ELISA antibody titers using recombinant *env*-gene products (baculovirus and *E. coli*), ELISA antibody titers do not correlate with virus-
25 neutralization and with protection.

Morikawa et al., Virology (1991) 183:288, report the expression of FIV *gag* protein in a baculovirus expression system.

SUMMARY OF THE INVENTION

Disclosed are recombinant feline immunodeficiency virus (FIV) vaccines
30 containing both a DNA sequence encoding FIV *gag* protein and a DNA sequence encoding FIV *env* protein. In one embodiment, the vaccines include a gene expression system for expressing FIV *gag* and *env* proteins from the DNA sequences encoding the FIV *gag* and *env* proteins. Among the gene expression systems available are the *E. coli*, yeast, chinese hamster ovary cells and baculovirus
35 expression systems, with baculovirus being preferred. In an alternate embodiment, the vaccines include a replicating vector for expressing FIV *gag* and *env* proteins

from the DNA sequences encoding FIV *gag* and *env* proteins. Among the replicating vectors available are the herpes, pox, adeno, retro and paramyxo viruses with feline herpes virus being preferred as the replicating vector. Other available replicating vectors are the salmonella bacteria. Also disclosed are combined

5 mucosal/parenteral, mucosal/mucosal and parenteral/parenteral inoculation methods.

The present invention provides vaccines and methods of protecting animals from feline immunodeficiency virus (FIV) infection by vaccinating them with recombinant vaccines containing both a DNA sequence encoding FIV *gag* protein and a DNA sequence encoding FIV *env* protein each expressed by the baculovirus expression system. A combined parenteral/parenteral inoculation method is provided.

The present invention provides vaccines and methods of protecting animals from feline immunodeficiency virus (FIV) infection by vaccinating them with recombinant vaccines containing both a DNA sequence encoding FIV *gag* protein and a DNA sequence encoding FIV *env* protein each expressed by the baculovirus expression system in conjunction with recombinant vaccines containing both the DNA sequence encoding FIV *gag* protein and the DNA sequence encoding FIV *env* protein each expressed by feline herpes virus (FHV) vectors. A combined mucosal/parenteral inoculation method where a first inoculation via mucosal administration of the FHVFI*env* and FHVFI*gag* vaccine, followed by a second inoculation via parenteral administration of the recombinant baculovirus vaccine is also provided.

The present invention provides vaccines and methods of protecting animals from feline immunodeficiency virus (FIV) infection by vaccinating them with recombinant vaccines containing both a DNA sequence encoding FIV *gag* protein and a DNA sequence encoding FIV *env* protein each expressed by feline herpes virus (FHV) vectors. A combined mucosal/mucosal inoculation method where a first inoculation via mucosal administration of the FHVFI*env* and FHVFI*gag* vaccine, followed by a second inoculation via mucosal administration of the FHVFI*env* and FHVFI*gag* vaccine is provided.

More particularly, this aspect of the invention provides a combined mucosal/parenteral inoculation where the first inoculation is nasal and the second is intramuscular.

35 Most particularly, the invention provides mucosal administration of *gag* and *env* protein via the FHV vector to a cat, followed by subcutaneous administration of

gag and *env* protein produced by recombinant baculovirus.

Immunization kits suitable for use in the method of the invention are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 illustrates a schematic diagram of the construction of pVLF*Igag*, the vector used for baculovirus expression of the FIV *gag* gene product;

 Figure 2 illustrates a schematic diagram of the construction of pVLF*Ienv*, the vector used for baculovirus expression of the FIV *env* gene product;

 Figure 3 illustrates the steps in the construction of pGCF*Igag*, the vector
10 used to produce the live feline herpesvirus expressing the *gag* gene product (FHV-
F*Igag*);

 Figure 4 illustrates the steps in the construction of pGCF*Ienv*, the vector
 used to produce the live feline herpesvirus expressing the *env* gene product (FHV-
F*Ienv*);

15 Figure 5 illustrates a Western blot demonstrating the expression of FIV *gag*
 and *env* proteins using the baculovirus expression system; and

 Figure 6 illustrates a Western blot demonstrating the expression of FIV *gag*
 and *env* proteins by recombinant feline herpesviruses.

Detailed Description of the Invention

20 Both the FIV *gag* and *env* sequences used in the construction of recombinant
 baculovirus were obtained from an infectious proviral clone of feline
 immunodeficiency virus, FIV-14. This clone was originally derived from the wild-
 type Petaluma strain. The location and sequences of the genes encoding *gag* and
 env of FIV, as well as the protein sequences encoded thereby, are known and may be
25 isolated following techniques well known in the art (see, e.g., Talbott et al., Proc.
 Natl. Acad. Sci. (1989) 86:5743; Olmsted et al., Proc. Natl. Acad. Sci. (1989)
 86:2448). Numbering of the sequence is as described in Olmsted RA et al., Proc.
 Natl. Acad. Sci. USA (1989) 86:8088 (GenBank accession number M25381).

 Alternatively, one can use these published sequences for *gag* and *env* to
30 chemically synthesize the genes using an apparatus designed for this purpose
 following techniques well known in the art. Using recombinant techniques the
 genes encoding *gag* and *env* of FIV, or immunogenic portions thereof, have been
 transformed into plasmids and thus the genes can be conveniently, and in the
 transfer vectors of the invention preferably are, obtained from such a source.

35 Exemplary plasmids are pVLF*Igag** (encoding the *gag* gene)(Fig. 2) and pVLF*Ienv*
 (encoding the *env* gene)(Fig. 1). General subcloning procedures were followed

as described in Sambrook, et al., Molecular Cloning: A Laboratory Manual (2nd edition, 1989), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

The baculovirus expression vector system (BEVS) is well known in the art as a convenient recombinant gene expression system which in some cases has been shown to produce large quantities of heterologous protein. Briefly, BEVS uses expression vectors to insert heterologous genes into the baculovirus genome at a location such that the gene will be expressed under the control of the baculovirus regulatory elements. The recombinant baculovirus is allowed to infect a cultured insect cell line, where the heterologous protein is expressed.

Several groups using BEVS have expressed the surface glycoproteins of two retroviruses. In addition, Morikawa et al., Virology (1991) 183:288-297, report the expression of FIV *gag* in the baculovirus system. BEVS is reviewed in detail by Luckow, V.A., Cloning and expression of heterologous genes in insect cells with baculovirus vectors, in Recombinant DNA Technology and Applications, Eds. C. Ho, A. Prokop, and R. Bajpai (1990) McGraw-Hill, New York, and Luckow, V.A. & Summers, M.D., Bio/Technology 6:47-55 (1988), which are incorporated by reference. For the construction of the recombinant baculovirus of the invention we use the method described in detail in the latter publication.

While there are a large number of baculovirus species known, the preferred virion for use in BEVS are *Autographa californica* nuclear polyhedrosis virus, also known as AcNPV or AcMNPV. AcNPV infects over 30 varieties of Lepidoptera cells, the preferred host being the *Spodoptera frugiperda* cell line Sf9.

The foreign genes to be inserted into baculovirus use plasmids which contain a cloning site flanked by baculovirus DNA. The cultured host cell is cotransfected with this plasmid and genomic wild-type baculovirus DNA, which recombines to produce a viral genome carrying the heterologous gene. The methods and conditions by which cotransfection occurs are well known in the art. Examples include calcium phosphate co-precipitation (Graham, F. L. and van der Eb, A. J., Virology 52:456-467 (1973)), protamine (Wienhues, U., et al, DNA (NY) 6:81-89 (1987)), lipofectin (Biotechniques 11:310-312) and electroporation (Mann, S. G. and King, L. A., J. Gen. Virology 70:3501-3505 (1989)). We use the calcium phosphate method, outlined in more detail below, which is the preferred transfection method to produce the recombinant baculovirus of the invention.

Typically, the heterologous gene is targeted for insertion into the polyhedron gene, a gene which is not essential for replication or production of extracellular virus. This is generally accomplished by including in the plasmid transfer vector

5 baculovirus DNA encoding the polyhedron promoter and sequences of 3' and 5' DNA flanking the polyhedron promoter. Foreign genes are then inserted into the transfer vector downstream of the promoter using recombinant DNA techniques known in the art. A wide variety of suitable transfer vectors are known and would be suitable for use in embodiments of the invention. Among these are pACYMI, pEV55, pAC373, pACRP, pEVIV, pEV51, and pVL941; the preferred vector is pVL941. Further, both the *gag* and *env* genes could be inserted into a single virus using several of the multiple cloning site vectors which are available. Examples of suitable multiple site vectors include p2XIVVI-X3, pXIVVI, pSyn nWTVI (as described in Gene 100:131-137 (1991)), or pACVC2, as described in Protein Engineering 1:359-366 (1987).

10 The recombinant baculovirus of the invention can be identified by visual screening followed by DNA dot blot hybridization, cell affinity techniques, plaque hybridization, or other techniques known in the art. The recombinant baculovirus of the invention may also include proteins for which there are chromogenic and/or enzymatic substrates for ease of identification and purification. The preferred method is visual screening of recombinants since recombinants lack the occlusion bodies characteristic of wild-type baculovirus; visual screening is a technique which is well known in the art. The occlusion-lacking morpholytes are then picked and placed into a 96-well plate for DNA dot blot hybridization. This hybridization technique is well known in the art of recombinant DNA technology and requires no special mention. It is preferred to purify and more fully characterize several of the viruses corresponding to the strongest hybridization signals.

20 The recombinant baculovirus of the invention may be propagated in any number of continuously cultured insect cell lines, most typically *Anticarsa gemmatilis* (soybean caterpillar), *Bombyx mori* (silkworm), *Estigmene acrea* (saltmarsh caterpillar), *Heliothis virescens* (tobacco budworm), *Leucania separata*, *Lymantria dispar* (gypsy moth), *Malacasoma disstria* (forest tent caterpillar), *Mamestra brassicae* (cabbage worm), *Manduca sexta* (tobacco hornworm), *Plutella*

30 *zyllostella* (diamond-back moth), *Spodoptera exigua* (beet armyworm), and *Spodoptera littoralis*. The preferred insect cell line for the propagation of the recombinant baculovirus of the invention is *Spodoptera frugiperda* Sf-9. In addition to the baculovirus expression system, other gene expression systems which can be used in the present invention include E. coli, yeast, and CHO expression systems.

35 An alternative system for introducing immunogens into hosts are well-known replicating vectors such as feline herpes virus. Genes to be expressed in a feline

herpes virus vector are placed under the control of a heterologous promoter and are inserted into the herpes virus genome. Typically, the gene to be expressed will be inserted into the thymidine kinase locus, allowing selection of recombinant viruses by loss of thymidine kinase function. The recombinant feline herpesvirus is then
5 used to directly infect the animal, where replication allows expression of the inserted genes. Alternate replicating viruses which can be used include pox-, adeno-, paramyxo-, and retroviruses. In addition, salmonella bacteria can be used as replicating vectors.

The immunogen can be prepared in vaccine dose form by well-known
10 procedures including the direct inoculation of plasmids containing DNA encoding the immunogen ("naked DNA vaccines").

A vaccine prepared utilizing the glycoproteins of the instant invention can consist of fixed host cells, a host cell extract, or a partially or completely purified FIV glycoprotein preparation from the host cells or produced by chemical synthesis.
15 The FIV glycoprotein immunogen prepared in accordance with the present invention is preferably free of intact FIV virus. Thus, the vaccine immunogen of the invention is composed substantially entirely of the desired immunogenic FIV polypeptides displaying FIV antigenicity.

The vaccine can then be administered parenterally or mucosally. For
20 parenteral administration, such as intramuscular or subcutaneous injection, the immunogen may be combined with a suitable carrier, for example, it may be administered in water, saline or buffered vehicles with or without various adjuvants or immunomodulating agents including aluminum hydroxide, aluminum phosphate, aluminum potassium sulfate (alum), beryllium sulfate, silica, kaolin, carbon, water-
25 in-oil emulsions, oil-in-water emulsions, muramyl dipeptide, bacterial endotoxin, lipid X, *Corynebacterium parvum* (*Propionibacterium acnes*), *Bordetella pertussis*, polyribonucleotides, sodium alginate, lanolin, lysolecithin, vitamin A, saponin, liposomes, levamisole, DEAE-dextran, blocked copolymers or other synthetic adjuvants. Such adjuvants are available commercially from various sources, for
30 example, Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.). Another suitable adjuvant is Freund's Incomplete Adjuvant (Difco Laboratories, Detroit, Michigan). Other vaccines may be prepared according to methods well known to those skilled in the art as set forth, for example, in I. Tizard, *An Introduction to Veterinary Immunology*, 2nd ed. (1982), which is incorporated herein by reference.
35 For parenteral administration the proportion of immunogen and adjuvant can be varied over a broad range so long as both are present in effective amounts. For

example, aluminum hydroxide can be present in an amount of about 0.5% of the vaccine mixture (Al_2O_3 basis). On a per dose basis, and depending on the purity and immunogenicity of the antigen, the concentration of the immunogen can range from about 1.0 μg to about 100 mg per cat. The preferred concentration of
5 immunogen and volume to be administered will vary depending on the age and weight of the host, as well as other factors known by those knowledgeable in the art of vaccination techniques. For example, in cats a preferable range is from about 10 μg to about 1.0 mg; a suitable dose size is about 0.5-5 ml, preferably about 1.0 ml. Accordingly, a dose for injection, for example, would comprise 1 ml containing 1.0
10 mg of immunogen in a mixture with 0.5% aluminum hydroxide. Comparable dose forms can also be prepared for parenteral administration to immature mammals, but the amount of immunogen per dose may be smaller, in kittens for example, about 0.25 to about 1.0 ml per dose.

For mucosal administration the immunogen may be combined with a suitable
15 carrier, for example, water, saline, or buffered vehicles. In addition, various immunomodulating agents known in the art may be added to specifically enhance mucosal immune response. Such agents include cholera toxin or parts thereof, DEAE-dextran, interleukins (e.g. IL-5), LT toxin of *E. coli*, Shiga toxin, and other toxins from gram-negative organisms. Once formulated, such vaccines may be
20 introduced at any mucosal surface, typically and most conveniently into the nares and/or oro-pharynx using devices suitable for this purpose e.g., dropwise with a small nasal cannula, by aerosolization, etc. The concentration of immunogen as well as dose size for mucosal administration is similar to that used for parenteral administration.

25 Vaccination can be accomplished following a two dose regime as disclosed in co-pending and similarly assigned Patent Application WO 9208427 which is herein incorporated by reference. As disclosed in WO 9208427, in a first embodiment the two dose regimen comprises a first dose administered to a mucosal membrane, as outlined above, followed by a second dose administered parenterally. Following this
30 aspect of the invention, the second dose is administered at some time following the first, mucosal, inoculation. The time period which should lapse between the first mucosal inoculation and the second later-in-time, parenteral, inoculation depends on the age, weight, health, etc. of the host, the virus against which protection is sought, the immunogenicity of the respective vaccines, etc. It is well recognized that, under
35 normal conditions, the mucosal administration would normally, but not always, comprise a replicating agent, i.e. live recombinant virus or bacteria, allowing

antigens to gain access to the immune system. In addition, the second parenteral inoculation may consist of either a replicating or non-replicating agent. However, this protocol is not the exclusive mode of delivering an antigen as non-replicating antigens may be delivered to the mucosal immune system, e.g by microsphere
5 encapsulation of antigens. These and other factors are well known in the art and the determination of the weight and relative importance of any of these factors is within the routine consideration of one skilled in the art.

The dual procedure is effective against viruses which show a compartmentalized immunogenic response i.e., viruses which gain entry into the
10 host via a mucosal site but which also replicate systemically. The dual procedure is also effective against organisms, such as feline immunodeficiency virus, which replicate predominantly at either the mucosal site or the parenteral site.

This dual dose regime of the invention may also be accomplished by administering the two vaccine components simultaneously at their appropriate sites.
15 Administration to both sites at precisely the simultaneous time is recognized by those skilled in the art to be unlikely and, thus, the time period for simultaneous administration includes the delay which may be experienced as, for example, the host is prepared for the second dose, the second dose is prepared for administration, the host is observed for signs of distress after the administration of the first dose,
20 etc. Thus, when following the simultaneous administration method of the invention, a cat, for example, would receive a suitable dose of an appropriate vaccine formulation intranasally and/or orally and simultaneous intramuscular vaccination with an appropriate vaccine.

When vaccines are administered following the dual procedures of the
25 invention, the vaccines may contain the same immunogenic component manufactured or presented in the same or different vector or formulated in the same or different vehicle. It is believed that a simultaneous dual administration as just described provides stimulation to both immunogenic compartments and thus diminishes the need for the second, later-in-time, dose.

30 In a second embodiment, vaccination with the FIV vaccines of the invention may also be accomplished following traditional regimes. Typically, a first dose is administered at a parenteral site or, more rarely in the case of some vaccines, at a mucosal site. The choice of site of first inoculation depends on the animal to be treated as well as the availability and suitability of the vaccine for that site.
35 Following a suitable period of time, a second dose is administered. Traditionally, the second dose is administered at the same site as the first, i.e., if the first is

mucosal the second is mucosal. The decision as to which site is appropriate for initial administration, the need for and the period of time prior to a second dose, the dose to be given, etc., are all factors known and understood by one skilled in the art of mammalian vaccination.

5 For example when vaccinating cats, the first dose can be given at 6-10 weeks of age. The second dose of the vaccine then should be administered some weeks after the first dose, for example, about 2 to 4 weeks later. Alternatively, the vaccine can be administered as a single 1 ml dose, for example, at about 6-10 weeks of age. However, a two dose regimen is considered preferable for the most effective
10 immunization of the cat. Annual revaccination is recommended. Adults may be revaccinated at any time. Kittens born to unvaccinated adults may be vaccinated at about 3-10 days, again at 4-6 months and yearly thereafter.

The FIV vaccines of the invention, as well as known vaccines, when administered following traditional techniques or when administered following the
15 dual method of co-pending Patent Application WO 9208427, may be combined with other vaccines for other diseases to produce multivalent vaccines. In addition, the vaccines of the invention, as well as known vaccines when administered following traditional techniques or when administered following the dual method of the invention, may also be combined with other medicaments, for example, antibiotics.

20 A "virus-like particle" is a particle produced by baculovirus infected insect cells which resembles an immature FIV particle in density (upon chromatography) and in size, shape, and ring structure (upon EM analysis).

The molecular biological, virological, and cell culture techniques described in the construction of both the recombinant baculoviruses and the recombinant feline
25 herpes viruses expressing feline immunodeficiency antigens are within the skill of the art. Literature that describes these techniques would include Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (2nd edition, 1989); Ausubel, et al., Current Protocols in Molecular Biology (1987); O'Reilly, et al., Baculovirus Expression Vectors: A Laboratory Manual (1992); Practical Molecular Virology (Collins, ed., 1991); Culture of Animal Cells: A Manual of Basic Technique (Freshney, ed., 2nd edition, 1989); J. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1972); D. A. Morrison, Transformation and Preservation of Competent Bacterial Cells by Freezing, *Methods Enzymol.* 68:326-331 (1979); J.
30 Perbal, A Practical Guide to Molecular Cloning, John Wiley & Sons (1984), and M. D. Summers and G. E. Smith, Texas Agricultural Experimental Bulletin No. 1555

(1987), all of which are incorporated by reference. Except where noted, all restriction enzymes, chemicals, and materials, or their equivalents, are readily available from commercial vendors. Endonuclease restriction follows manufacturer recommendations.

5 Without further elaboration, it is believed that one skilled in the art can, using the preceding description, practice the present invention to its fullest extent. The following detailed examples describe how to construct the various recombinant vaccines of the invention and/or perform the various processes of the invention and are to be construed as merely illustrative, and not limitations of the preceding
10 disclosure in any way whatsoever. Those skilled in the art will promptly recognize appropriate variations from the procedures both as to reactants and as to reaction conditions and techniques.

Materials and Methods

Cells, viruses, and plasmids.

15 The parent baculovirus *Autographica californica* nuclear polyhedrosis virus (AcNPV), E2 strain, is obtained from Max Summers (Texas A & M University). The *Spodoptera frugiperda* cell line, Sf-9, is obtained from the American Type Culture Collection (CRL 1711). Plasmid pSP72 was obtained from Promega Biotec. Plasmid p3CL-DHFR was obtained from Fred Homa (The Upjohn Company). Plasmid
20 pGC113 was obtained from Jack Nunberg and is described by Nunberg et al., J. Virol. (1989) 63:3240.

Plasmid p3CL-DHFR is a pUC18-based vector constructed for the expression of heterologous genes in eucaryotic cells. It uses the human cytomegalovirus (CMV) immediate promoter and leader (sequences -1140 to +74 relative to the start site of
25 transcription) to drive transcription. A 550 base pair BamHI-BglII fragment containing bovine growth hormone sequences is used to supply the polyadenylation functions. The vector contains unique HindIII and SalI sites between the CMV promoter and bGH polyadenylation sequences for insertion of foreign genes.

Cell line FIV-14 was derived from the wild type Petaluma strain (Olmsted et
30 al., Proc. Natl. Acad. Sci. (USA) 86:8088 (1989). The cells, parent viruses, and recombinant viruses are propagated by methods described in detail in Texas Agricultural Experimental Bulletin No. 1555 (1987).

Plasmid pVL941 is described by Luckow, V.E. and M.D. Summers, Virology 170:31-39 (1989).

35 Animals

Eighteen specific pathogen-free cats of approximately 12 weeks of age are

used in all experiments. Animals are housed in a containment facility either in three groups of six animals each and were fed cat food and water *ad libitum*.

Western blot analysis.

For western blot analysis, aliquots from each fraction of the gradients are separated using standard SDS PAGE. Following electrophoresis, proteins are electroblotted onto 0.22 um nitrocellulose following the procedure of Towbin, H., et al, Proc. Natl. Acad. Sci. USA 76:4350-4354 (1979). Unoccupied sites on the nitrocellulose are blocked by sequential incubation in PBS containing 5% non-fat dry milk (NFDM). Blots are incubated with the primary antibody and color development carried out as described by Hink, W.F., et al, Biotechnology Progress 7:9-14 (1991). Convalescent cat sera from experimentally FIV-infected cats was used to detect *gag* and *env* proteins on Western blots.

Preparation of antigen for use as vaccine.

Spodoptera frugiperda (Sf-9) cells in spinner flasks are infected at a cell density of 1×10^6 cells/ml at a multiplicity of infection of 5 plaque forming units (pfu)/cell. The cells are infected with the following recombinant viruses: either AcNPVFIgag* or AcNPVFIenv, or co-infected with AcNPVFIgag* and AcNPVFIenv. Each culture is harvested at 66 hours post infection. Infected cells are separated from the culture medium by low speed centrifugation and stored frozen until used.

FHV recombinants expressing either FIV *gag* or *env* genes are grown in CRFK cells and harvested 3 days post-infection. After clarification to remove cell debris, supernatant fluid is titered and stored at -70°C before use. Vaccine is formulated to contain 10^5 - 10^7 pfu/ml.

Laboratory Assays.

p24 levels in cats are measured using a commercially available kit (IDEXX, Portland, ME). p24, a group-specific antigen of FIV, is found in the blood of persistently-infected animals and hence can be used as a test for viraemia. Standardization in our laboratory indicated that within the context of known positive and negative sera, O.D. values of ≥ 0.25 are positive for viraemia.

PCR analysis of cat PBMC's was performed using two pairs of nested primers during two rounds of amplification on the thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). The sequences of the oligonucleotide primers used in PCR and as probes for Southern blots were selected from FIV sequence data (Talbot et al., Proc. Natl. Acad. Sci. (1989) 86:5743 and Olmsted et al., Proc. Natl. Acad. Sci. (1989) 86:2448). Each round of amplification consisted of 30 cycles (Round 1: 94°C for 1 min., 55°C for 1.5 min., 72°C for 2 min., 0.1 ml final cycling volume; Round 2; 94°C

for 1 min, 55°C for 1.5 min, 72°C for 1 min., 0.05 ml final cycling volume) where round 1 begins with genomic DNA and round 2 amplifies a 0.01ml sample from each of the 1st round reactions.

Samples were then analyzed on a 0.9% agarose gel in TBE buffer by loading
5 0.05ml of PCR round 2 product. The 804-bp DNA product was visualized by ethidium bromide staining and confirmed by Southern blot.

Following electrophoresis, transfer of DNA to nitrocellulose was done according to standard procedures (Sambrook, J., E.F. Fritsch, and T. Maniatis, (1989) *Molecular Cloning: A Laboratory Manual* (2nd Ed.) Cold Spring Harbor
10 Laboratory Press, Cold Spring Harbor, N.Y.). The filter hybridization was performed using Amersham's 3' - oligolabeling system with nonradioactive detection using enhanced chemiluminescence (ECL). In the ECL reaction, horseradish peroxidase catalyzes the oxidation of luminol to detect probes which are 3'-tailed with fluorescein-dUTP and hybridized to target sequences on filters. Using the
15 probe PJD 83: 5'- ACA TCC CCC TGA TGC TCC CAG ACC ATT ACC -3' (Talbot et al., *Proc. Natl. Acad. Sci.* (1989) 86:5743) [SEQ ID NO:3] at a concentration of 10 ng/ml, the membrane was hybridized at 55°C for 2 hours.

Example 1 Expression of FIV *gag* and *env* genes.

A. Cloning of *gag* gene into recombinant baculovirus expression vector

20 To construct a recombinant baculovirus expressing the FIV *gag* gene product, the infectious proviral clone pFIV14 is digested with Hinc II and Eco RV to release a 1450 base fragment (bases 599-2049) containing the *gag* gene. This fragment is subcloned into the vector pSP72, digested with Sma I and dephosphorylated with calf intestinal phosphatase (CIP). The resulting intermediate plasmid (designated
25 D4) is then digested with Bgl II and Bam HI to release the *gag*-containing 1450 base fragment, which is then ligated into the baculovirus expression vector pVL941 (Talbot et al., *Proc. Natl. Acad. Sci.* (1989) 86:5743) digested with Bam HI and dephosphorylated with calf intestinal phosphatase. The resulting vector is designated pVLFI_{gag} (See Fig. 1).

30 The design of pVLFI_{gag} results in an arrangement that allows the naturally-occurring FIV *gag-pol* frameshift to produce a fusion protein consisting of the FIV *gag* joined to the baculovirus polyhedron gene product. To eliminate this frameshift product, stop codons in all three reading frames are inserted immediately following the *gag* reading frame. PCR is used to produce a "replacement" 3' end for the *gag*
35 gene, containing the desired stop codons. PCR primers (DAD-1: 5'- CCATGGAATTCTACCTATTTATAAATCCAATAGTTCTCCTC-3'[SEQ ID NO:1],

DAD-2: 5'-GCAATGGCCACCTTAAGCCAGAAAG-3' [SEQ ID NO:2]) are used to amplify a 389 base portion of FIV-14 containing the engineered stop codons. The PCR fragment is digested with Eco RI and the 125 base fragment containing the "replacement" *gag* 3' end is isolated. This fragment is then ligated into the *gag*-
5 containing D4 plasmid which has been digested with Eco RI (and dephosphorylated) to remove the fragment containing the natural 3' end. The resulting plasmid, designated D4*, is then placed into pVL941 in the same manner as described above for D4 (see Fig. 1) to yield the baculovirus vector pVLF*Igag**. The section of the gene which is PCR-amplified is sequenced to confirm the presence of the desired
10 stop codons.

B. Cloning of *env* gene into recombinant baculovirus expression vector

A vector to express FIV *env* in baculovirus is constructed by first digesting pFIV-14 with Ase I and Nde I to remove a 2649 base fragment (bases 6257-8906) containing the FIV *env* gene. After treatment with Klenow to fill in the ends, the
15 fragment is subcloned into pSP72 digested with Sma I and dephosphorylated. This intermediate plasmid, designated B1, is digested with Bam HI and Bgl II (partial digest) to remove a ~2660 base fragment which is ligated into pVL941, digested with Bam HI and dephosphorylated. The resulting plasmid is designated pVLF*Ienv* (See Fig. 2).

20 C. Baculovirus transformation

Plasmids pVLF*Igag** and pVLF*Ienv* are co-transfected with the wild-type baculovirus DNA into Sf9 cells using standard baculovirus expression vector techniques (Texas Agricultural Experimental Station Bulletin No. 1555 (1987). Recombinant viruses are selected on the basis of their occlusion-negative phenotype.
25 Five individual plaques are picked for each construct and plaque-purified five times. The viral stocks are tested for expression of the heterologous FIV gene by first infecting a 60 mm dish of Sf9 cells at an estimated multiplicity of infection of 10. At 60 hrs. post infection the conditioned media and the cells are harvested and tested for expression of FIV proteins by Western blot analysis. Serum from a cat
30 experimentally infected with FIV is used to probe the Western blot. Positive clones producing the highest levels of protein are selected to make viral stocks.

D. FIV *env* and *gag* expression (baculovirus)

Sf9 cells growing in suspension culture are used to produce larger quantities of protein for animal experiments. Suspension cells are grown in spinner flasks in
35 media consisting of supplemented Grace's media (Gibco BRL) containing 10% fetal calf serum. The media is also supplemented with amphotericin B (2.5 ug/ml),

penicillin (10U/ml), and streptomycin (10 ug/ml). Cells are infected at a density of 1×10^6 cells/ml with an m.o.i. of 10. Sixty-six hours post infection the cells are harvested by centrifugation. Expression is confirmed by Western blot analysis prior to use. Fig. 5 shows a Western blot of cells or media from 1) CRFK cells infected with FHVFIgag (for comparison) (lane 1), 2) uninfected Sf9 cells (neg. control) (lane 2), 3) Sf9 cells infected with AcNPVFIenv (lane 3), 4) Sf9 cells infected with AcNPVgag* (lane 4), 5) Sf9 cells infected with AcNPVgag (lane 5). The blot is probed with FIV-reactive antisera from an experimentally infected cat. Infected cells are stored frozen at -20°C .

10 E. Cloning of gag and env genes into recombinant feline herpes virus

The same FIV sequences used for expression of FIV gag and env genes in baculovirus are used to construct recombinant feline herpesviruses expressing FIV gag and env gene products.

The gene containing FIV gag coding sequences is removed from the FIV proviral clone FIV-14 by digestion with Hinc II and Eco RV (See Fig. 3). This 1450 base fragment is subcloned into the vector p3CL-DHFR which has been digested with Hind III, the ends filled in with Klenow, and dephosphorylated. The resulting plasmid, p3CLFIgag is then digested with Eco RI (partial) and Bgl II to liberate a fragment containing the entire transcription unit, which consists of the CMV immediate early promoter, the FIV gag gene, and the bovine growth hormone polyadenylation signal. This fragment is subcloned into the plasmid pGC113 (Nunberg JH et al., J. Virol. (1989) 63:3240) at the Hind III site (both insert and vector blunt-ended with Klenow) to give the plasmid pGCFIgag. The transcription unit is flanked by FHV thymidine kinase gene sequences to allow homologous recombination into the FHV genome, and is missing ~450 bases from the middle of the thymidine kinase gene in order to attenuate the virus (absent from pGC113 as described in Nunberg JH et al., J. Virol. (1989) 63:3240).

A similar strategy is used to produce a transfer plasmid for expressing FIV env (See Fig. 4). The coding sequence for FIV env is removed from FIV-14 by digestion with Ase I and Nde I and subcloned into pSP72 digested with Pvu I and dephosphorylated to produce the plasmid designated F5. The env gene is removed from plasmid F5 with Hind III and Xho I and subcloned into p3CL-DHFR digested with Hind III and Sal I, yielding the plasmid p3CLFIenv. The fragment containing the CMV promoter, FIV env gene and bGH polyadenylation sequences is removed by digestion with Eco RI and Pvu II, blunt-ended with Klenow, and subcloned into the Hind III site of pGC113 (filled-in with Klenow) to give the plasmid pGCFIenv.

F. FIV *env* and *gag* expression (FHV)

Recombinant FHV's expressing FIV *gag* or *env* are produced essentially as described in Nunberg JH et al., J. Virol. (1989) 63:3240). This involves using homologous recombination to replace the native thymidine kinase gene with the
5 deleted thymidine kinase gene containing the FIV gene transcription unit. CRFK cells are co-transfected using a standard calcium phosphate precipitate transfection protocol with equal amounts of either pGCFI*gag* or pGCFI*env* and purified FHV (UT88) DNA. After 100% CPE is reached (3-5 days), the resulting virus pool is serially diluted and re-plated on CRFK cells in the presence of 100 ug/ml thymidine
10 arabinoside (araT) to select for thymidine kinase-negative viruses. Eight plaques are picked from each transfection, amplified on CRFK cells, and viral DNA is prepared. The viral DNA is analyzed by restriction digest with Eco RI and subsequent Southern blot analysis using both FIV-specific and tk-specific DNA probes. DNA-positive clones are analyzed for expression of FIV proteins by Western
15 blot analysis of infected cell lysates.

Fig. 6 shows the expression of FIV *gag* and *env* proteins by recombinant feline herpes viruses. CRFK cells are infected with three different FHVFI*gag* clones (lanes 1,2,3), four different FHVFI*env* clones (lanes 5,6,7,8), or the parental UT88 virus as a negative control (lanes 4,9). Cell lysates are analyzed by Western blot
20 analysis using an FIV-reactive antisera from an experimentally infected cat. Recombinant FHV's expressing FIV *gag* or *env* are selected on the basis of maximum protein expression, plaque-purified an additional two times, and used to produce viral stocks.

Example 2 Vaccination and challenge experiments

25 Eighteen specific pathogen free cats of approximately 12 weeks of age are used in this experiment. Animals are housed in containment facilities in three groups of six animals each and fed dried cat food and water *ad libitum*.

A. Vaccination groups

1. Combined Mucosal/Parenteral Vaccination.

30 After a period of acclimation, animals in Group I (n=6) are intranasally inoculated with 0.5ml. of FHVFI*gag* and 0.5ml. of FHVFI*env* (1 ml total). This is accomplished by using a syringe to which a small nasal canula has been attached. Animals are gently restrained and approximately 0.25 ml is dropped into each nostril and 0.5 ml placed towards the back of the oropharynx. Each separate
35 preparation contains $10^{6.5}$ TCID₅₀ per ml. Approximately three weeks later animals are revaccinated subcutaneously with $10^{6.5}$ Sf9 cells, suspended in saline,

which 72 hours previously had been infected, at an MOI of 1, with recombinant baculoviruses with FIV *gag* and *env* insertions.

2. Combined Parenteral/Parenteral Vaccination.

Animals in Group II (n=6) are inoculated subcutaneously with $10^{6.5}$ Sf9 cells suspended in saline, which 72 hours previously had been inoculated, at an MOI of 1, with recombinant baculoviruses with FIV *gag* and *env* insertions. Approximately three weeks later these animals are revaccinated with a similar recombinant baculovirus preparation.

3. Combined Mucosal/mucosal vaccination

After a period of acclimation, animals are intranasally inoculated with 0.5ml. of FHVFI_{gag} and 0.5ml. of FHVFI_{env} (1 ml total). This is accomplished by using a syringe to which a small nasal canula has been attached. Animals are gently restrained and approximately 0.25 ml are dropped into each nostril and 0.5 ml placed towards the back of the oropharynx. Each separate preparation contains $10^{6.5}$ TCID₅₀ per ml. Approximately three weeks later animals are revaccinated in a similar manner with the identical dose.

4. Control

Animals in Group III (n=6) are left as unvaccinated controls.

B. Challenge

Approximately two weeks after the last vaccination, animals in each of the three groups are challenged intravenously with a one ml dose of a 10^{-3} dilution of a cloned viral stock of the Petaluma isolate of FIV. There is no evidence of immunosuppression. Titration of this stock in susceptible cats using a similar route and inoculation amount has shown it to have a titer of 10000 ID50s.

Blood taken from these cats at 24 weeks post challenge is separated on Ficoll gradients to enrich for mononuclear cells. These cells are cultured at 10^5 per ml in RPMI 1640 with 10% fetal calf serum in the presence of 2 μ g Con A and 100 IU of recombinant human IL-2 per ml. After three days cells are spun down and resuspended in RPMI 1640 with 10% fetal calf serum and 100 IU of recombinant human IL-2 per ml. After 21 days in culture, cells are prepared for PCR analysis.

Cat peripheral blood mononuclear cells (PBMC's) are harvested on the 21st day of culture by centrifugation at 1500 x g. Cells are then washed with Hank's Balanced Salt Solution (HBSS). Genomic DNA is isolated from approximately 10^7 cells using the Turbogen Kit (Invitrogen Corporation, San Diego, CA.). Briefly, cells are lysed using a denaturing cationic detergent. Then detergent and denatured protein are removed via chloroform extraction. Genomic DNA is precipitated from

the aqueous phase with a low salt buffer and ethanol is then dissolved in TBE buffer and stored at 4°C until analysis by PCR (described in Laboratory Assays).

C. Results

Table 1 shows that four of six animals in the control group are viremic at 24 weeks, whereas none of the animals inoculated parenterally/parenterally or mucosally/parenterally are positive on either the ELISA antigen assay or PCR. This indicates that these vaccination schedules successfully prevent persistent viraemia with FIV. It is expected that mucosal/mucosal vaccination will achieve similar results.

10 Example 4 Vaccination Kit

A kit containing the vaccines for administration and instruction on the dual mucosal/parenteral administration of vaccines, parenteral/parenteral or mucosal/mucosal administration of vaccines, as described herein, may be produced as a safe and cost efficient means of supplying a vaccine. The vaccination kit contains vaccines in single or multiple use vials, ampules, or other suitable containers. For mucosal administration the formulated vaccine may be in containers or in an aerosolized form. For parenteral use, single dose syringes containing a suitable efficacious dose for the host mammal represent an easy and rapid way in which administration is accomplished. Alternatively, the kit may contain the mucosal and parenteral vaccines in multi-use vials or other suitable containers, and may be supplied ready-to-use or require some additional preparation or mixing prior to use. An instruction sheet is included which more fully describes the included vaccine(s) to be administered and their formulation, the order and timing of inoculation, as well as additional factors of concern to the practitioner.

For cats, as an example, such a vaccination kit may contain a one ml, single dose, mucosal formulation containing FHVFlenv and FHVFIgag (2×10^5 pfu/vaccine) to be administered 0.5 ml to each nostril. The parenteral formulation includes one ml of AcNPVenv and AcNPVgag (2×10^5 pfu/vaccine) for intramuscular administration.

The foregoing detailed description is given for clearness of understanding only, and no unnecessary limitations should be inferred therefrom, as modifications within the scope of the invention will be obvious to those skilled in the art.

Table 1: FIV antigen and PCR results on 24 week samples.

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Cat number and vaccination history	Assay Result*	
	FIV antigen assay	PCR result on 21 day cultured PBMC
XXD3 CONTROL	-	-
XXE2 CONTROL	+	+
XXH3 CONTROL	-	+
XXA2 CONTROL	+	+
XXH2 CONTROL	-	+
XXS3 CONTROL	-	-
XXQ3 BACULO REC.	-	-
XXE3 BACULO REC.	-	-
XWL2 BACULO REC.	-	-
XXH1 BACULO REC.	ND	ND
XWG2 BACULO REC.	ND	ND
XWE3 BACULO REC.	ND	ND
XXL1 FHV REC.	-	-
XXB1 FHV REC	-	-
XWX3 FHV REC	ND	ND
XWP3 FHV REC	-	-
XWR2 FHV REC	-	-
XWR1 FHV REC	-	-

*Antigen presence assayed on the IDEXX ELISA and PCR analysis performed according to the text. + signifies a positive result - signifies a negative result. ND =not done (cultured cells were lost due to lack of growth and were not available for testing at the end of the experiment).

SEQUENCE LISTING

- 5 (1) GENERAL INFORMATION:
- (i) APPLICANTS: The Upjohn Company,
Wardley, Richard C. and David E. Lowery
- 10 (ii) TITLE OF INVENTION: Feline Immunodeficiency Virus Vaccine
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
- 15 (A) ADDRESSEE: The Upjohn Company, Corp. Intellectual
Property Law
(B) STREET: 301 Henrietta Street
(C) CITY: Kalamazoo
(D) STATE: Michigan
20 (E) COUNTRY: USA
(F) ZIP: 49001
- (v) COMPUTER READABLE FORM:
- 25 (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
- 30 (A) APPLICATION NUMBER: 4761.P CP
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- (viii) ATTORNEY/AGENT INFORMATION:
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(C) TELEX: 224401
- (2) INFORMATION FOR SEQ ID NO:1:
- 45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
50 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- 55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
CCATGGAATT CTACCTATTT ATAAATCCAA TAGTTCCT C 41
- (2) INFORMATION FOR SEQ ID NO:2:
- 60 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
65 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
5 GCAATGGCCA CCTTAAGCCA GAAAG 25
(2) INFORMATION FOR SEQ ID NO:3:
10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
15 (ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
20 ACATCCCCCT GATGCTCCCA GACCATTACC 30

CLAIMS

We claim:

1. A recombinant feline immunodeficiency virus (FIV) vaccine comprising a
5 DNA sequence encoding FIV *gag* protein and a DNA sequence encoding FIV *env* protein.
2. A vaccine in accordance with claim 1 further comprising a gene expression system for expressing FIV *gag* and *env* proteins from said DNA sequences encoding
10 FIV *gag* and *env* proteins.
3. A vaccine in accordance with claim 2 wherein said gene expression system is selected from the group consisting of E. coli, yeast, chinese hamster ovary cells and baculovirus.
15
4. A vaccine in accordance with claim 3 wherein said gene expression system is a baculovirus expression system.
5. A vaccine in accordance with claim 1 further comprising a replicating vector
20 for expressing FIV *gag* and *env* proteins from said DNA sequences encoding FIV *gag* and *env* proteins.
6. A vaccine in accordance with claim 5 wherein said replicating vector is selected from the group consisting of herpes, pox, adeno, retro and paramyxo viruses
25 and salmonella bacteria.
7. A vaccine in accordance with claim 6 wherein said recombinant replicating vector is feline herpes virus.
- 30 8. A recombinant feline immunodeficiency virus (FIV) vaccine comprising a DNA sequence encoding FIV *gag* protein and a DNA sequence encoding FIV *env* protein wherein said DNA sequences encoding FIV *gag* and *env* proteins are expressed by both a gene expression system and a replicating vector.
- 35 9. A vaccine in accordance with claim 8 wherein said gene expression system is selected from the group consisting of E. coli, yeast, chinese hamster ovary cells and

baculovirus and said replicating vector is selected from the group consisting of herpes, pox, adeno, retro and paramyxo viruses and salmonella bacteria.

10. A vaccine in accordance with claim 9 wherein said gene expression system is
5 a baculovirus expression system and said replicating vector is feline herpes virus.

11. A method of vaccinating a cat against feline immunodeficiency virus (FIV) comprising:
administering to the cat a recombinant feline immunodeficiency virus (FIV) vaccine
10 comprising a DNA sequence encoding FIV *gag* protein and a DNA sequence encoding FIV *env* protein.

12. A method in accordance with claim 11 wherein said vaccine comprises DNA sequences encoding FIV *gag* and *env* proteins expressed by a gene expression
15 system.

13. A method in accordance with claim 12 wherein said gene expression system is selected from the group consisting of E. coli, yeast, chinese hamster ovary cells and baculovirus.
20

14. A method in accordance with claim 13 wherein said gene expression system is a baculovirus expression system.

15. A method in accordance with claim 11 wherein said vaccine comprises DNA
25 sequences encoding FIV *gag* and *env* proteins expressed by a replicating vector.

16. A method in accordance with claim 15 wherein said replicating vector is selected from the group consisting of herpes, pox, adeno, retro and paramyxo viruses and salmonella bacteria.
30

17. A method in accordance with claim 16 wherein said recombinant replicating vector is feline herpes virus.

18. A method in accordance with claim 11 comprising administering a first dose
35 of said vaccine followed by a second dose of said vaccine.

19. A method in accordance with claim 18 wherein said first and second doses of said vaccine are administered parenterally.
20. A method in accordance with claim 19 wherein said first and second doses of said vaccine comprise DNA sequences encoding FIV *gag* and *env* proteins expressed by a baculovirus expression system.
21. A method in accordance with claim 18 wherein said first and second doses of said vaccine are administered mucosally.
22. A method in accordance with claim 21 wherein said first and second doses of said vaccine comprise DNA sequences encoding FIV *gag* and *env* proteins expressed by FHVFI*env* and FHVFI*gag*.
23. A method in accordance with claim 18 wherein said first dose of said vaccine is administered mucosally and said second dose of said vaccine is administered parenterally.
24. A method in accordance with claim 23 wherein said first dose of said vaccine comprises DNA sequences encoding FIV *gag* and *env* proteins expressed by FHVFI*env* and FHVFI*gag* and said second dose of said vaccine comprises DNA sequences encoding FIV *gag* and *env* proteins expressed by a baculovirus expression system.
25. A vaccination kit comprising:
(a) a recombinant vaccine comprising DNA sequences encoding both FIV *gag* and *env* proteins expressed by FHV for mucosal administration; and
(b) a recombinant feline immunodeficiency virus (FIV) vaccine comprising a DNA sequence encoding FIV *gag* protein and a DNA sequence encoding FIV *env* protein expressed by a baculovirus expression system for parenteral administration.
26. A vaccination kit comprising a recombinant feline immunodeficiency virus (FIV) vaccine comprising a DNA sequence encoding FIV *gag* protein and a DNA sequence encoding FIV *env* protein expressed by a baculovirus expression system for parenteral administration.

27. A vaccination kit comprising a recombinant vaccine comprising DNA sequences encoding both FIV *gag* and *env* proteins expressed by FHV for mucosal administration.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86 C07K14/155 A61K39/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF CLINICAL MICROBIOLOGY, vol.31, no.9, 1993 pages 2350 - 2355 VERSCHOOR, E.J. ET AL. 'Expression of Feline Immunodeficiency Virus gag and env precursor proteins in Spodoptera frugiperda cells and their use in immunodiagnosis'	1-27
X	WO,A,93 08836 (INSTITUT PASTEUR) 13 May 1993 see claims 2,3,18,20,32,33,49,50	1,11
Y	EP,A,0 576 092 (AKZO N.V.) 29 December 1993 see page 4, line 21 - line 23; claims	1-27



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

* & * document member of the same patent family

Date of the actual completion of the international search

7 August 1995

Date of mailing of the international search report

18.08.95

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Chambonnet, F

INTERNATIONAL SEARCH REPORT

Inter national Application No
PCT/US 95/03998

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,90 13573 (IDEXX CORPORATION) 15 November 1990 see page 4, line 26 - line 28; claims 1-6,8-12 ---	1-27
A	WO,A,94 06471 (PITMAN-MOORE INC.) 31 March 1994 see the whole document ---	1
A	WO,A,94 06921 (VIAGENE INC.) 31 March 1994 see claims 4-6,9,10 ---	1
A	WO,A,90 15141 (APPLIED BIOTECHNOLOGY INC.) 13 December 1990 see claims 1-10 ---	1
A	WO,A,94 03621 (AKZO N.V.) 17 February 1994 see the whole document -----	1

INTERNATIONAL SEARCH REPORT

Int. ional application No.

PCT/US 95/ 03998

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 11-24
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 11-24 are directed to a method of treatment of the animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 95/03998

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9308836	13-05-93	AU-A-	2800392	07-06-93
		CA-A-	2122263	13-05-93
		EP-A-	0613378	07-09-94
		JP-T-	7501052	02-02-95
EP-A-0576092	29-12-93	AU-B-	4149693	06-01-94
		CA-A-	2099069	27-12-93
		HU-A-	68442	28-06-95
		JP-A-	6070761	15-03-94
		NZ-A-	247971	26-05-95
WO-A-9013573	15-11-90	CA-A-	2016183	08-11-90
		EP-A-	0471752	26-02-92
		JP-T-	4507094	10-12-92
WO-A-9406471	31-03-94	AU-B-	4826493	12-04-94
		CA-A-	2143187	31-03-94
		EP-A-	0661999	12-07-95
WO-A-9406921	31-03-94	AU-B-	5138293	12-04-94
		CA-A-	2142325	31-03-94
		EP-A-	0662139	12-07-95
WO-A-9015141	13-12-90	AU-A-	5848390	07-01-91
		CA-A-	2033175	02-12-90
		EP-A-	0431128	12-06-91
		JP-T-	4500312	23-01-92
WO-A-9403621	17-02-94	AU-B-	4702293	03-03-94
		EP-A-	0606452	20-07-94
		JP-T-	6511392	22-12-94

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